

# Preparation of Bovine Immunoglobulins and Free Secretory Component and Their Specific Antisera

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## Abstract

The preparation of bovine IgG1, IgG2, IgM, secretory IgA and free secretory component is described. The methods require a minimum of equipment and utilize four basic procedures: precipitation, electrophoresis, gel filtration, and DEAE chromatography. The electrophoretic and immunochemical techniques described for assaying the purity of the various preparations are sensitive and those utilizing radial or immunodiffusion are inexpensive. The immunoelectrophoretic and disc electrophoretic patterns for each preparation are diagnostic and highly reproducible. Methods are described for the preparation of monospecific antisera against each preparation and a polyvalent antiserum against bovine immunoglobulins.

In addition, the presence of 7 S IgM in bovine serum, and proteolytic digestion fragments and aggregates of IgG in whey, are described. The occurrence of these immunoglobulin units helps to explain the complicated immunoelectrophoretic patterns often observed in bovine body fluids and also the multiple ring phenomena obtained during radial diffusion assay for immunoglobulins in the cow.

## Introduction

There is a growing need for diagnostic antisera against the recognized classes and subclasses of bovine immunoglobulins in the fields of dairy, food, and veterinary science research.

Received for publication August 6, 1971.

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<sup>3</sup> Mention of a trade name, distributor, or manufacturer is for identification only and implies no endorsement of the product or its manufacturer.

<sup>4</sup> We acknowledge W. J. Stein for assistance in the preparation of antisera in rabbits and guinea pigs.

Currently, antisera and proteins of the quality needed for the preparation of such antisera are not commercially available.

We report the preparation of bovine IgG1, IgG2, IgM, secretory IgA (SIgA) and free secretory component (FSC). Methods are described for evaluating the purity of these proteins, for preparing monospecific antisera against each protein and for preparing polyvalent antisera for bovine immunoglobulins. In addition, new data are presented on bovine 7 S IgM and on proteolytic digestion fragments and aggregates in milk whey. The proposal to call glycoprotein-a (16) bovine FSC (7, 29) has been followed in this paper.

## Materials and Methods

*Ion-exchange and gel filtration chromatography.* Ion-exchange chromatography was performed on DEAE-Sephadex A-50<sup>3</sup> equilibrated with a 0.1 M Tris, pH 8.3 buffer containing 0.05 M NaCl. Gradient elution was performed by adding 240 ml of starting buffer to each of the first two chambers of a Varigrad<sup>3</sup> and filling the third chamber with 240 ml of the same buffer containing 0.22 M NaCl. The molarity at various points during elution was determined by comparing the conductivity of the eluant with an established standard curve for the buffer system employed.

Recycling gel filtration was performed on upward flowing columns (5 × 100 cm) containing Superfine Sephadex G-200<sup>3</sup> equilibrated with 0.1 M Tris buffer, pH 8.6 which contained 0.9 M NaCl, 0.02% sodium azide, and 0.003 M EDTA. Protein in the eluant was monitored at 282 mμ and specific proteins monitored by single radial diffusion.

*Fractional precipitation of serum and lacteal secretions.* Serum and whey were dialyzed six hours against cold running tap water (10 C) and then 48 to 72 hr against three changes of distilled water at 4 C to effect precipitation of the euglobulins. The water soluble fraction of serum and whey was precipitated once with 33% saturated ammonium sulfate by the method of Campbell et al. (10). The resuspended precipitate was considered the water soluble immunoglobulin fraction (WSIF) of

serum or whey. The whey supernatant, although still rich in immunoglobulins, was called the water soluble nonimmunoglobulin fraction (WSNF) and was used for the preparation of free secretory component. Fat was removed from colostrum and normal milk by centrifugation for 60 min at  $2,500 \times g$  after which the fat layer was removed with a spatula or the skim milk removed by insertion of an aspirator tube below the fatty layer. The caseins were removed by lowering the pH to 4.6 with 0.1 M HCl, incubating for 30 min, and centrifuging for 30 min at  $2,500 \times g$ .

**Dialysis and concentration of protein fractions.** Positive pressure dialysis and concentration were performed with membranes and units manufactured by the Amicon Corporation.<sup>3</sup> Dialysis of large volumes of serum and whey was carried out for 24 to 72 hr at 4 to 10°C by simple dialysis against large volumes of the appropriate buffer. Dialyzer tubing was supplied by Arthur H. Thomas, Inc.<sup>3</sup>

**Disc electrophoresis in acrylamide gel.** Analytical disc electrophoresis was carried out in 10 M urea by modification of the procedure described by Reisfeld et al. (30). The urea was first deionized by passing it over a mixed bed resin (Crystalab Cl-5<sup>3</sup>) until the conductivity was less than 10 to 20  $\mu$ mhos. All stock solutions were prepared in 10 M urea and had a shelf life of two weeks. A 4% acrylamide gel was used and samples were electrophoresed for 3 hr at 5.0 mamp/tube. Preparative zonal electrophoresis was performed on Pevikon C-870 blocks (Stockholms Superfosfat Fabrik<sup>3</sup>)  $30 \times 6 \times 1.5$  cm, for 30 hr at 1.5 mamp/cm in 0.05 M barbital buffer, pH 8.2 (1, 12).

**Immunodiffusion, single radial diffusion, and immunoelectrophoresis.** Microimmunodiffusion (41) and microimmunoelectrophoresis (33) were performed on  $7.62 \times 2.54$  cm microscope slides in 2% Ionagar 2. Electrophoresis was carried out for 90 min at 12 mamp/slide with a 0.05 M barbital buffer, pH 8.2. The maintenance of constant immunoelectrophoretic conditions among various runs was assured by periodic measurements of buffer conductivity.

Semi-quantitative single radial diffusion was performed as described by Fahey and McKelvey (13) and was used to detect precipitin reactions not apparent from immunodiffusion studies and to determine the distribution of specific proteins in the eluants from ion exchange and gel filtration columns.

**Immunization procedures.**<sup>4</sup> One-milliliter solutions of IgG1 or IgG2 containing 1 to 5 mg of protein were homogenized with equal volumes

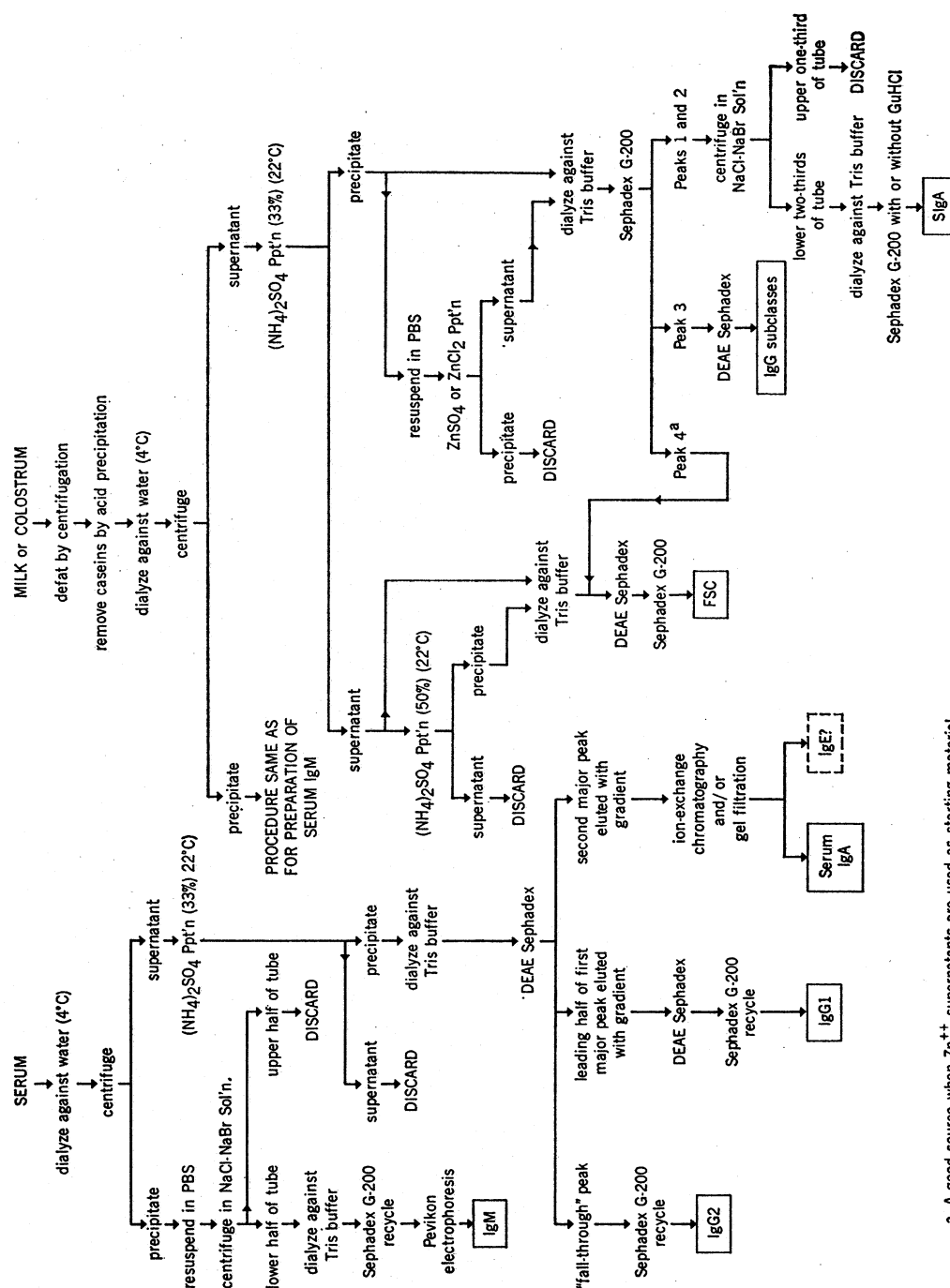
of Freund's complete adjuvant and 0.25 ml of this mixture injected into each foot pad of a rabbit. Animals were boosted 30 days later with an intravenous injection of 5 mg of the appropriate immunoglobulin. Animals were exsanguinated 7 to 10 days later and their sera prepared. Rabbits immunized similarly with IgM, SIgA, and free secretory component (FSC) were first rendered tolerant to IgG by the method of Spiegelberg and Weigle (36). Antisera to other proteins mentioned in this report (ribonuclease, lactoferrin, transferrin and serum albumin) were prepared similarly. The preparation of antiserum to  $\alpha$ -2 macroglobulin is discussed later.

The immunization procedure of Binaghi et al. (3) was used for the production of antisera in guinea pigs. Only female guinea pigs weighing over 425 g were selected.

**Specific adsorption procedures.** Antosera to IgG1 and IgG2 were rendered subclass specific by repeated reciprocal absorption with insoluble immunoabsorbents prepared from IgG2 and IgG1 by the method of Avrameas and Ternynck (2). Antibodies to light chain determinants and  $\gamma$ -chains were removed from the antisera prepared against FSC, SIgA, IgM and  $\alpha$ -2 macroglobulin using an insoluble immunoabsorbent prepared by the same method (2) from commercially prepared bovine  $\gamma$ -globulin (Gallard-Schlesinger<sup>3</sup>). Antibodies to  $\alpha$ -2 macroglobulin were removed from any of the above preparations by using an immunoabsorbent prepared in the same way from fetal bovine serum (Microbiological Associates<sup>3</sup>). Complete gelling of the fetal serum-glutaraldehyde complex is extremely pH dependent and in our hands works best at pH 4.1 after overnight chilling of the mixture.

## Results

**Preparation of serum immunoglobulins.** The euglobulin fraction from two liters of bovine serum was redissolved in 0.1 M Tris buffer, 0.15 M NaCl, pH 8.3. An equal volume of a sodium chloride-sodium bromide solution (0.195 molal NaCl, 7.60 molal NaBr), having a density of 1.48, was mixed with the euglobulin fraction and the mixture centrifuged for 24 hr at  $114,000 \times g$ . The lower layer (half of the tube volume) was collected and the lipoproteins in the upper layer discarded (Fig. 1). The lower layer was dialyzed against the Tris buffer used for gel filtration. This fraction was applied to a column of G-200 Sephadex and the results illustrated in Figure 5. The first protein peak was collected while the subsequent



a. A good source when  $Zn^{++}$  supernatants are used as starting material.

Fig. 1. Flow sheet summarizing the preparation of bovine immunoglobulins and free secretory component.

protein peaks containing 7 S IgG, 7 S IgM (to be discussed later) and albumin were either collected for other uses or discarded. The first peak was recycled twice and the leading portion collected (Fig. 5, Cycle III). Two hundred milligrams of this IgM-rich fraction were then

subjected to electrophoresis in Pevikon and IgM subsequently eluted from the first 10 cm of the block on the anodal side of the origin. IgM prepared by this method gave a single precipitin line on immunoelectrophoresis when tested with anti-whole bovine serum (Fig. 3), a

single sedimenting peak in the ultracentrifuge and a single electrophoretic zone at the stacking gel-separating gel interface (Fig. 4). Radial diffusion assay revealed the presence of 2-5% IgG.

The WSIF of serum was dialyzed against the starting buffer used for ion-exchange chromatography and 250 mg of this material were then applied to a  $12 \times 2.5$  cm column of DEAE-Sephadex A-50. The flow was adjusted to 60 ml per hour and the column was washed with the starting buffer. After all unadsorbed protein had been removed ("fall-through" peak, Fig. 1, and Peak 1, Fig. 2), the adsorbed protein was eluted with the NaCl gradient described under Materials and Methods. The elution profile is shown in Figure 2.

The "fall-through" fractions from several similar runs were pooled and rechromatographed to remove any more negatively charged molecules that might have been present in the individual "fall-through" fractions as a result of column saturation. Following rechromatography on DEAE-Sephadex, the aggregate "fall-through" peak was recycled on G-200 to remove any IgG polymers or small molecular weight contaminants. The 7 S protein obtained in this manner, labelled IgG2, gave a diagnostic slow precipitin arc on immunoelectrophoresis

(Fig. 3), precipitated with anti-IgG2 (Fig. 10B) and not anti-IgG1 (Fig. 9D and 10B) and moved faster toward the cathode than IgG1 on disc electrophoresis (Fig. 4). On immunoelectrophoresis, the anodal end of the precipitin line of purified serum IgG2 rarely extended beyond the origin and rarely showed the degree of charge heterogeneity seen among different IgG1 preparations (Fig. 3).

The first major protein peak appearing after the start of the gradient (Peak 2, Fig. 2) was rich in serum IgG1 although the descending portion also contained transferrin and serum IgA. Recycling gel filtration of the leading portion of this fraction yielded a preparation of serum IgG1 which formed a line of identity with colostral IgG1 when tested by immunodiffusion (Fig. 9D) and failed to react with anti-IgG2 (Fig. 10B). The immunoelectrophoretic (Fig. 3) and disc electrophoretic (Fig. 4) patterns of serum IgG1 were similar to those of colostral IgG1. IgG1 was also prepared from the third major peak shown in Figure 2 or from an equivalent fraction from colostral whey. This IgG1 had a much faster electrophoretic mobility than the serum or colostral IgG1 prepared from Peak 2 (Fig. 3).

Bovine serum IgA was also prepared from the third major peak (Peak 3, Fig. 2) obtained by gradient elution of WSIF by recycling gel filtration. A highly purified preparation of serum IgA, free of IgG1 and in a sufficiently useful quantity, was difficult to obtain by this method and others will be discussed.

*Preparation of immunoglobulins from colostral whey.* Three liters of colostrum were treated for the removal of fat and casein and the subsequent euglobulin and water soluble immunoglobulin fractions (WSIF) prepared. The IgM prepared from colostrum was immunologically identical to the one obtained from serum.

One gram of the appropriately equilibrated WSIF was applied to a  $5.0 \times 100$  cm G-200 column. The preparation was resolved into Peaks 1, 2, and 3 (Fig. 6). 7 S IgG is the protein eluted in Peak 3. The first and second peaks from several runs were pooled, the lipoproteins removed by centrifugation in NaCl-NaBr solution, as previously described, and the pooled fraction reappplied to the same column and recycled up to five times with care being taken to collect any 7 S material (Peak 3) that appeared. The 7 S material collected was pooled with the original Peak 3 and subsequently used for the preparation of IgG subclasses. After five cycles, the distribution

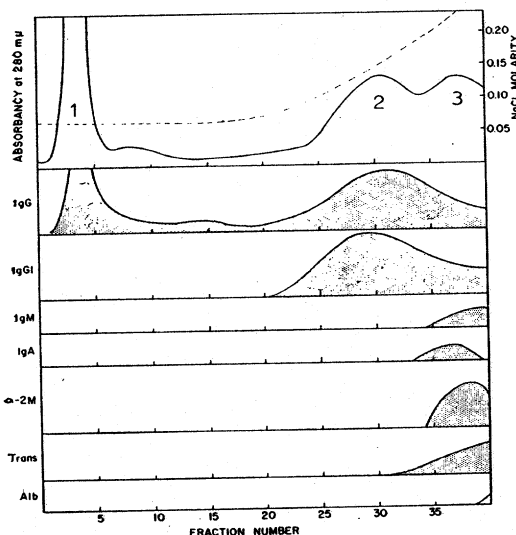


FIG. 2. Gradient elution profile of proteins (solid line) in the water soluble immunoglobulin fraction of bovine serum. Dotted line = molarity of NaCl. Distribution of specific proteins determined by radial diffusion is shown below. IgG = IgG1 + IgG2, Trans = transferrin, Alb = serum albumin. Peak 1 = "fall-through" peak.

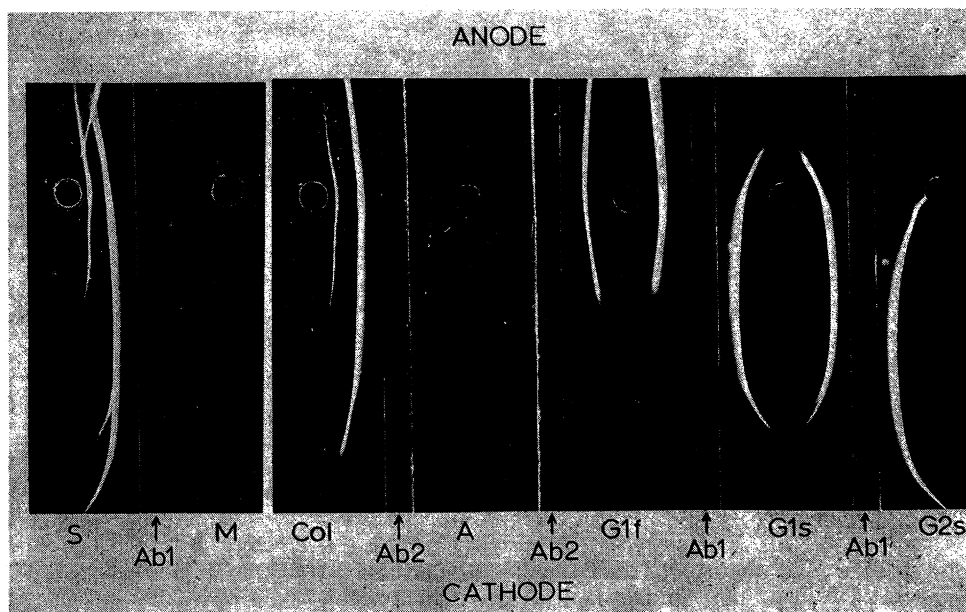


FIG. 3. Immunoelectrophoretic patterns of purified bovine immunoglobulins. Ab1 = antiserum to whole bovine serum, Ab2 = polyvalent antiserum to bovine immunoglobulins. S = serum; M = IgM; Col = colostral whey; A = colostral SIgA; G1f = IgG1 prepared from Peak 3, Figure 2; G1s = serum IgG1; G2s = serum IgG2.

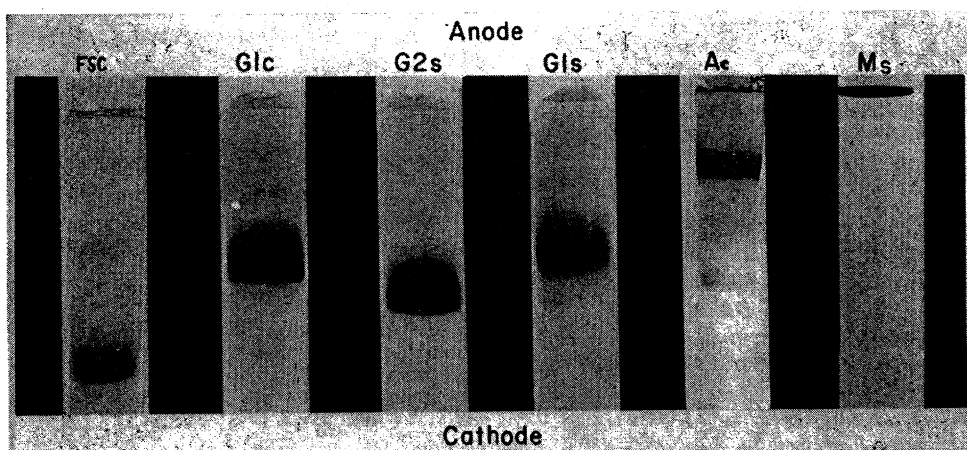


FIG. 4. Disc electrophoretic pattern of purified preparations of bovine immunoglobulins and free secretory component. Glc = colostral IgG1, G2s = serum IgG2, G1s = serum IgG1, Ac = colostral SIgA, Ms = serum IgM.

of the various immunoglobulins was determined by radial diffusion (Fig. 6, bottom). A sizeable portion of the protein in the original Peaks 1 and 2 was aggregated IgG with a sedimentation velocity of 9.5 S. The SIgA obtained in the second recycled peak routinely contained >75% SIgA as determined by single radial diffusion against anti-IgA and anti-IgG1 or anti-IgG2. The degree of IgG contamination is apparent in Figure 4.

The addition of  $\text{ZnCl}_2$  to a final concentration of 0.33 M precipitated 200 of 450  $\mu\text{g}$  of protein in a colostral whey sample. Preliminary immunoelectrophoretic studies indicated that SIgA was not removed from solution by this treatment. After dialysis of the supernatant,  $\text{ZnCl}_2$  was again added to raise the molarity to 0.33 over which range no additional precipitation occurred. Raising the molarity to 0.6  $\text{ZnCl}_2$  resulted in the precipitation of a

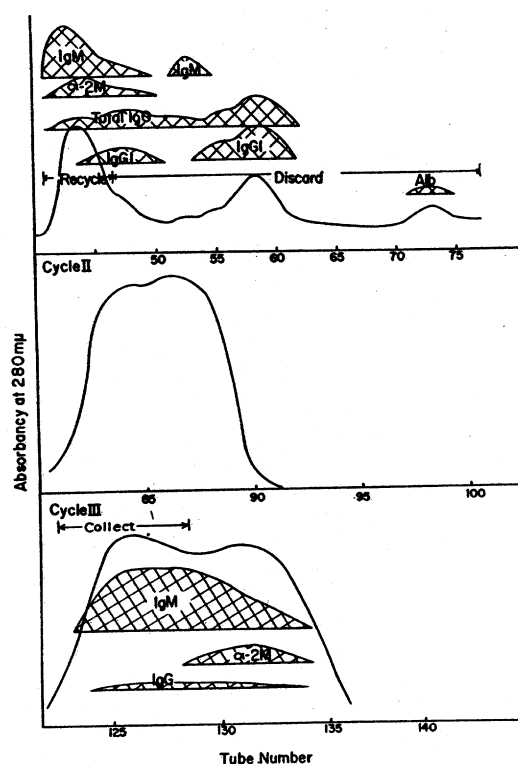


FIG. 5. Preparation of bovine serum IgM by recycling gel filtration. Top: protein elution profile of euglobulin fraction after a single pass on G-200. Cycle II and III; second and third passes of first peak from top profile. Distribution of specific proteins, determined by radial diffusion, is indicated.

large amount of protein. Immunoelectrophoretic analyses of equal concentrations (mg/ml of protein) of the precipitate, precipitate washings (washed with 0.6 M  $\text{ZnCl}_2$ ) and the supernatant gave the results shown in Figure 7 and suggested an enrichment of SIgA in the supernatant and first wash. Precipitation of the WSIF of whey with equal volumes of 0.05 M  $\text{ZnSO}_4$ , as described by Vaerman (40), also yields a supernatant enriched in IgA.

Gel filtration Peak 3 (Fig. 6) was dialyzed against the starting buffer used for ion-exchange chromatography and 250 mg of this protein were applied to a  $12 \times 2.5$  cm column of DEAE-Sephadex A-50. The results are similar to those from the WSIF of bovine serum in Figure 2 except for the absence of the third major peak and by the much smaller amount of protein in the "fall-through" peak (less than 10% of the total). The majority of the protein was eluted as colostral IgG1. Like the IgG1 fraction obtained by ion-exchange chromatography of the WSIF of serum, further

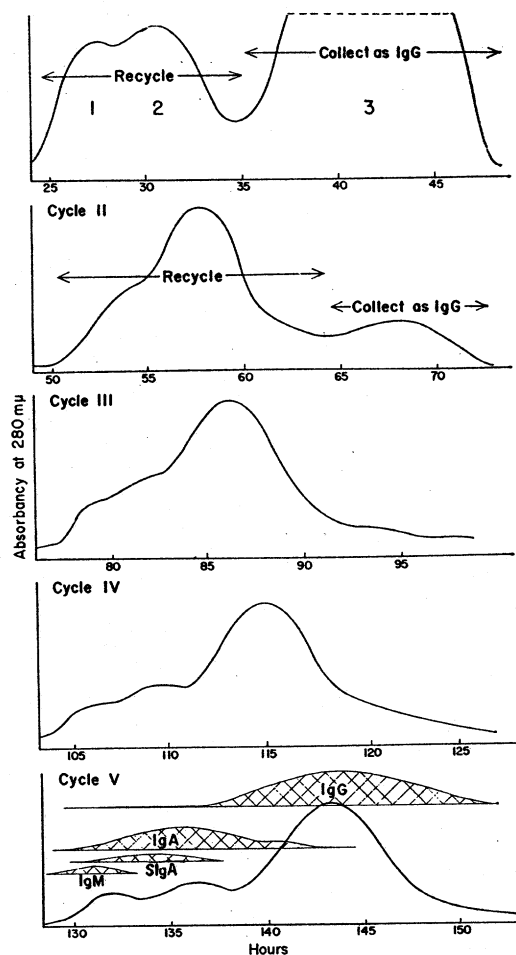


FIG. 6. Preparation of bovine SIgA from colostrum by recycling gel filtration. Top: protein elution profile of water soluble immunoglobulin fraction of colostral whey after a single pass on G-200. Cycles II to IV: successive passes of Peaks 1 and 2 from top profile. Distribution of IgM, IgA, SIgA and IgG is shown in Cycle V. Note the heterogeneity of colostral IgA, as described previously (7).

purification was accomplished by recycling gel filtration. The colostral IgG1 prepared by this method formed a line of identity with serum IgG1 when tested by immunodiffusion (Fig. 9D and 10B) while failing to precipitate with antisera specific for other immunoglobulins (Fig. 10). The disc electrophoretic pattern of colostral IgG1 was identical to that of serum IgG1.

*Preparation of free secretory component.* The WSNF of normal or colostral whey was equilibrated with the starting buffer used for DEAE-Sephadex chromatography. After the appearance of multiple "fall-through" peaks,

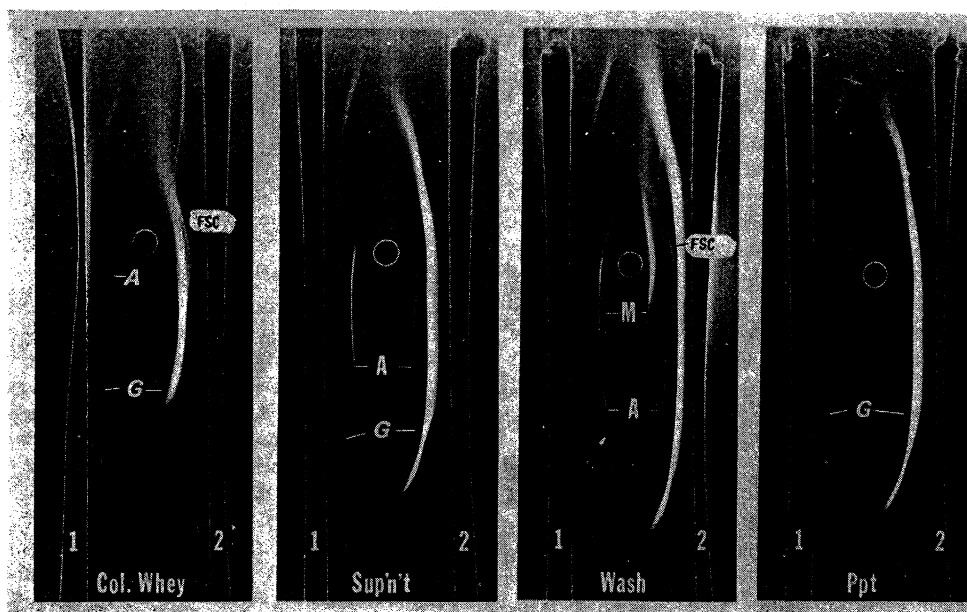


FIG. 7. Immunoelectrophoretic analyses of various fractions of colostrum whey after treatment with  $\text{ZnCl}_2$ . Cathode is at the bottom. 1 = unabsorbed antiserum to bovine IgA, 2 = polyvalent antiserum to bovine immunoglobulins. A = IgA, G = IgG, M = IgM. Col. whey = colostrum whey prior to treatment with  $\text{ZnCl}_2$ .

a gradient elution was initiated. The elution pattern obtained and the distribution of the various proteins are shown in Figure 8. The distribution of the various proteins in the multiple "fall-through" fraction will be discussed later. An alternate and more expedient procedure involves precipitation of FSC from the WSNF before chromatography on DEAE-Sephadex by the addition of an equal volume of saturated ammonium sulfate. This step removes much of the lactoferrin and smaller whey proteins in the WSNF of whey.

Free secretory component was eluted immediately after Peak 3 (Tubes 10 to 15, Fig. 8). The FSC-rich fractions from several runs were pooled and rechromatographed on DEAE-Sephadex. Free secretory component was separated from contaminating IgG and other unidentified smaller proteins by gel filtration on Sephadex G-200. As aggregates of FSC do occur in aqueous buffer, chromatography in guanidine hydrochloride ( $\text{GuHCl}$ ) was performed without loss of antigenicity or immunogenicity (7). Free secretory component prepared with or without  $\text{GuHCl}$  gave a single protein zone after disc electrophoresis (Fig. 4). This zone was slightly slower than that of lactoferrin (not shown) but faster than IgG2 electrophoresed under similar conditions. The immunoelectrophoretic

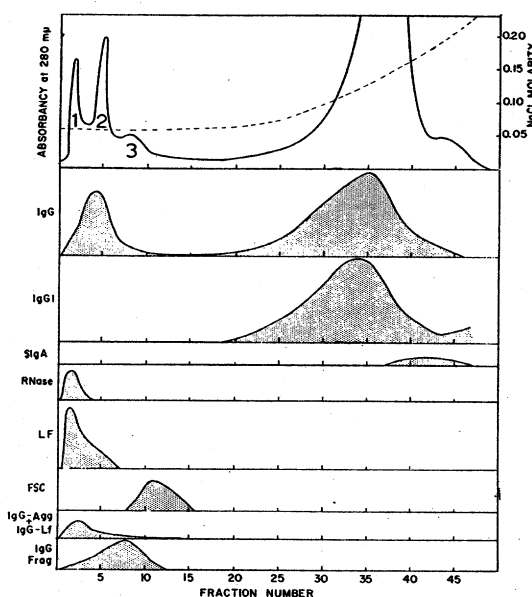


FIG. 8. Gradient elution profile of proteins (solid line) in the water soluble nonimmunoglobulin fraction of colostrum or normal whey. Dotted line = molarity of NaCl. Distribution of specific proteins determined by radial diffusion is shown below. IgG = IgG1 + IgG2, RNase = ribonuclease, Lf = lactoferrin, IgG-Agg = aggregated IgG2, IgG-Lf = IgG2-lactoferrin complexes, IgG Frag = proteolytic digestion fragments of bovine IgG. Peaks 1, 2, and 3 = "multiple fall-through" peak.

pattern of FSC was faster than that of SIgA, much less heterogeneous than SIgA (Fig. 10A), and much slower than that of lactoferrin (7).

Common light chain determinants were detected on all of the immunoglobulins whose preparation has been described (Fig. 9B). Free secretory component preparations contained no such light chain determinants (Fig. 9B). The immunodiffusion pattern obtained with anti-light chain antiserum showed a spur between reactions of IgG1 or IgG2 and IgM or SIgA.

The various preparation procedures described are summarized in Figure 1.

*Appearance of immunoglobulin fragments and aggregates in whey.* The distribution and identity of the various proteins in the multiple "fall-through" Peaks 1, 2 and 3 (Fig. 8) were established with a combination of immunochemical and biochemical techniques. Our finding will be published in greater detail elsewhere (6). The first peak was predominantly composed of lactoferrin but also contained ribonuclease, aggregated IgG and a lactoferrin-IgG2 aggregate. Peak 2 also contained some IgG2 aggregates but was particularly rich in 7 S IgG2. Aggregated IgG2 and 7 S IgG2 gave separate precipitin lines on immunoelectrophoresis, the aggregate forming a diffuse arc near the well (Fig. 9A). Peak 3 was predominantly composed of the less positively charged IgG2 molecules and the proteolytic digestion fragments Fc and Fab. In addition, a 5 S immunoglobulin fragment (mol wt = 110,000) was also present in Peaks 1-3. This fragment also formed a precipitin arc that was distinct from 7 S IgG2 and aggregated IgG2. The arc produced by the 5 S fragment was much sharper than the one produced by the aggregated IgG2 (Fig. 9A). The distribution of the various aggregates and fragments in the collection tubes encompassing Peaks 1-3 could be determined by immunodiffusion (Fig. 9C).

*Detection of 7 S IgM in bovine serum.* Radial diffusion assay of the globulin fraction of bovine serum (Fig. 5) indicated a discontinuous distribution of IgM. The last fraction having IgM specificity was eluted immediately before the bulk of the IgG. The molecular weight of this fraction, estimated by gel filtration, was 200,000. When this IgM-rich fraction was tested with antiserum specific for IgM, a line of partial identity was formed with the 19 S IgM in a lyophilized, reconstituted preparation of serum IgM (Fig. 9E). When tested with whole fresh serum, part of the line continued past the major IgM precipitin line and formed a precipitin line near the well in which

fresh serum had been added numerous times. This indicates that 7 S IgM is present in small amounts in normal serum. The precipitin line nearest the peripheral well to which the lyophilized, reconstituted IgM had been added, is due to aggregated (32 S) IgM. A faint corresponding line could also be seen in normal serum. The immunoelectrophoretic pattern of the 7 S IgM protein also differed from that of the principal IgM arc of bovine serum (Fig. 9 F). Although not shown, 7 S IgM formed a precipitin line with antiserum to light chains.

*Production of rabbit and guinea pig antisera to bovine immunoglobulins and free secretory component.* A summary of the results obtained by immunizing rabbits with bovine immunoglobulins and related proteins is presented in Table 1. Using the methods described under Materials and Methods, strong precipitating antisera to bovine immunoglobulins, assayed by immunodiffusion and immunoelectrophoresis, were readily obtainable in rabbits. Variations in specificity were routinely observed, particularly among antisera to SIgA. IgG, particularly IgG1, and  $\alpha$ -2 macroglobulin

TABLE 1. Results of immunizing rabbits with bovine immunoglobulins. Number of usable precipitating antisera obtained against the protein administered per the number of rabbits immunized.

Protein preparation administered	IgG tolerant <sup>a</sup>	AD <sup>b</sup>	IgG non-tolerant <sup>a</sup>	AD <sup>b</sup>
IgG1	.....	.....	7/10	0/10
IgG2	.....	.....	1/10	0/10
IgM, iv <sup>c</sup>	2/2	0/2	2/6 <sup>d</sup>	4/6
IgM, sc <sup>c</sup>	.....	.....	5/5	0/5
IgA	4/4	0/4	7/9	1/9
$\alpha$ -2 Macroglobulin	.....	.....	1/3 <sup>d</sup>	2/3
Free secretory component	2/2	0/2	2/2	0/2

<sup>a</sup> Tolerance to IgG (36) was successfully produced in 13 of 24 rabbits used for the production of antisera to bovine immunoglobulins and other bovine proteins not shown above.

<sup>b</sup> AD = Anaphylactic death.

<sup>c</sup> Challenging or booster injection given intravenously or subcutaneously.

<sup>d</sup> Because animals died of shock, evaluation of antiserum was not undertaken.



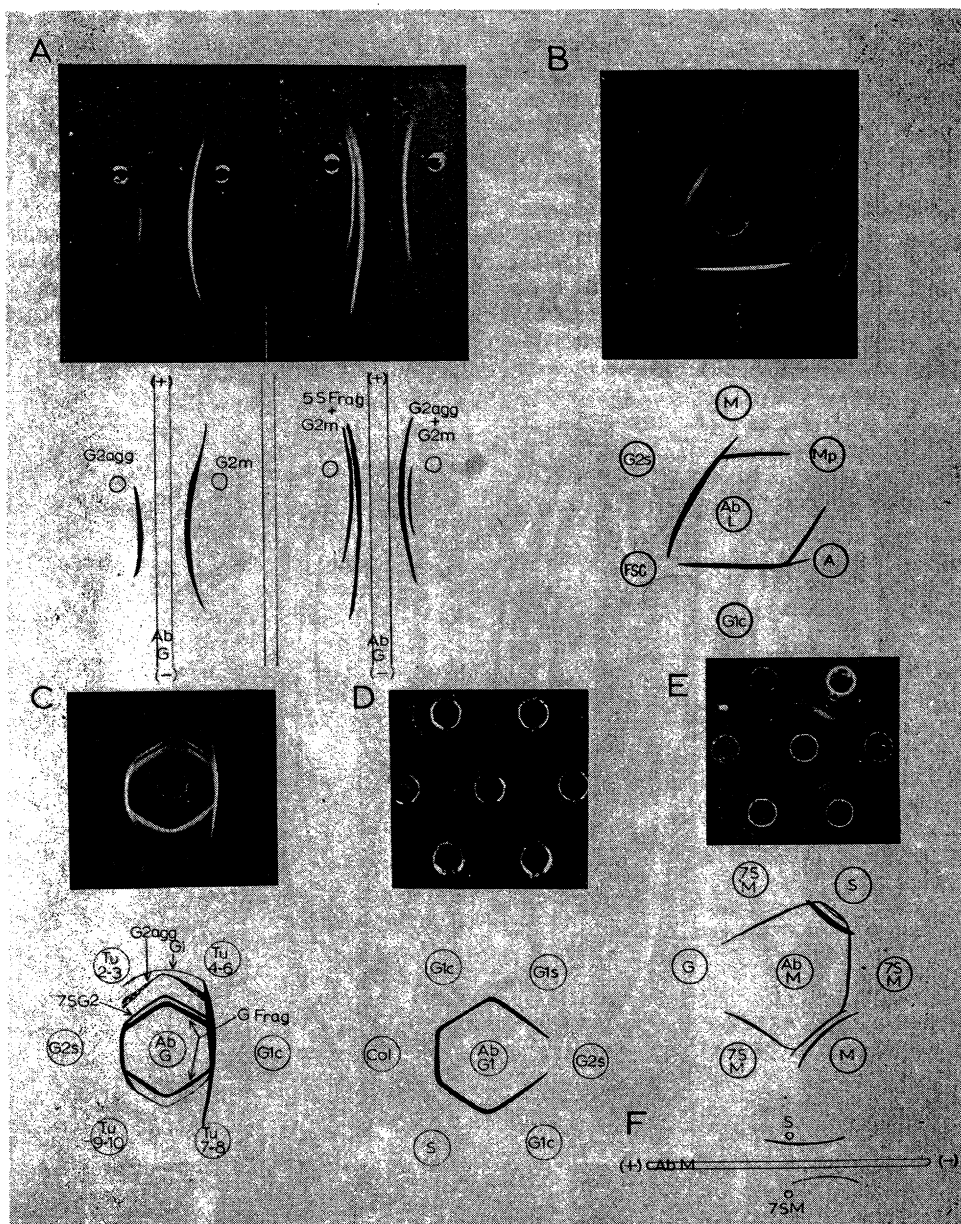


FIG. 9. Immunelectrophoretic and immunodiffusion analyses of various immunoglobulins and immunoglobulin fragments. Following symbols are used universally: G2s = serum IgG2, G1s = serum IgG1, M = IgM, G1c = colostral IgG1, A = SIgA, G = IgG1 + IgG2, S = serum. Ab = preceding any of the above denotes antiserum to that protein.

A. Immunelectrophoretic analyses of an aggregate and a digestion fragment of IgG2 from whey. Drawing below. G2agg = aggregated IgG2, G2m = 7 S IgG2, 5 S Frag = 5 S digestion fragment of IgG.

B. Immunodiffusion analysis of bovine immunoglobulins and free secretory component tested with an antiserum to light chains (Ab-L). Drawing below. Mp = nonimmunoglobulin control.

C. Immunodiffusion analyses of multiple "fall-through" peak shown in Figure 6. Drawing below. G2agg = aggregated IgG2, G Frag = primarily Fe and Fab fragments of IgG2 and IgG1. Tu = designates tube numbers used in Figure 6.

D. Immunodiffusion analyses of antiserum to bovine IgG1. Drawing below. Col = colostrum.

E. Immunodiffusion analyses of 7 S IgM (7 S M). Drawing below.

F. Immunelectrophoretic analyses of 7 S IgM (7 S M). Drawing only.

appeared to be the most antigenic in rabbits of the materials administered. For this reason the technique of Spiegelberg and Weigle (36) for producing tolerance to IgG was employed when the preparation of antisera to SIgA and IgM was undertaken. Rabbits tolerant to IgG produced antisera to SIgA and IgM that required little or no absorption with IgG immuno-adsorbents although such antisera were generally weaker than those produced against SIgA and IgM in nontolerant animals. None of the four antisera to SIgA prepared in tolerant rabbits demonstrated easily detectable antibodies to FSC (Fig. 10). However, anti-

sera produced against colostral or lacrimal SIgA in non-tolerant rabbits did detect FSC as well as SIgA (Fig. 10A). Some rabbits immunized with lacrimal SIgA detected additional antigenic determinants not detected in antisera raised to colostral SIgA (7).

While seven of ten rabbits immunized with IgG1 detected antigenic determinants found only on IgG1, only one animal of ten immunized with IgG2 detected IgG2-specific determinants. For this reason, antisera to bovine IgG2 were raised in guinea pigs. Eighty per cent of all guinea pigs studied to date responded to IgG2-specific determinants when immunized with

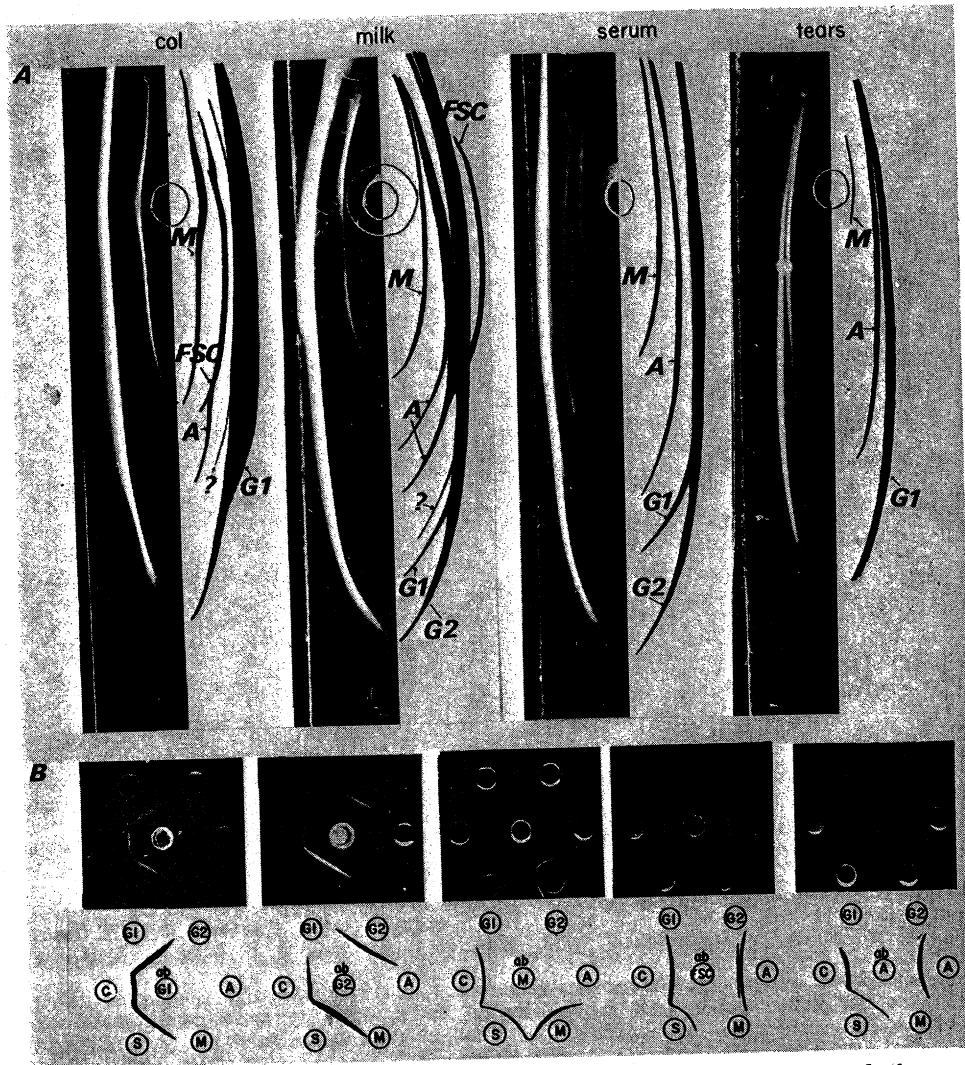


FIG. 10. Specificity of polyvalent antiserum to bovine immunoglobulins; A, and the specificity of five monospecific antisera B. Universal symbols used: G1 = colostral IgG1, G2 = serum IgG2, A = IgA, M = IgM, S = serum, ? = unidentified precipitin arc, C or Col = colostral whey. Ab = preceding any of the above denotes antiserum specific for that protein.

IgG2. The IgG1 immunizations listed in Table 1 are the total of both immunizations with serum IgG1 and colostral IgG1. No differences were found between the specificities of antisera prepared against serum and colostral IgG1. Immunization with colostral IgG1 is preferable to avoid the production of antibodies to highly antigenic transferrin which could contaminate serum IgG1 preparations.

Strong precipitating antisera to bovine IgM were obtained by a modification of the method of immunization described. Serious problems were encountered, particularly in nontolerant animals, when the IgM preparation used for immunization contained sizeable amounts of  $\alpha$ -2 macroglobulin. Four of six nontolerant rabbits immunized via the foot pads with IgM died of anaphylaxis when challenged intravenously 30 days later. None of the animals challenged subcutaneously were lost to anaphylactic shock (Table 1). The role of  $\alpha$ -2 macroglobulin in the anaphylactic deaths was surmised from experiments in which rabbits were immunized with  $\alpha$ -2 macroglobulin from fetal serum. Two out of three died when challenged intravenously 30 days later (Table 1). Curiously, IgG tolerant rabbits immunized with IgM and challenged intravenously did not die from anaphylactic shock (Table 1).

Antisera to FSC (Fig. 10B) were prepared equally well in tolerant and nontolerant rabbits. The double precipitin lines formed with the SIgA preparation in Figure 10B, using this antiserum, correspond to FSC and SIgA.

Absorption of rabbit and guinea pig antisera with glutaraldehyde linked immunoabsorbents was performed as described in Materials and Methods.

Polyvalent antisera to bovine immunoglobulins have proven particularly useful in qualitative assay of various body fluids (7) (Fig. 10), in carrying out qualitative studies of immunoglobulin synthesis (9), and for evaluating the purity of immunoglobulin preparations. The preparation of such polyvalent antisera by immunization of rabbits with whole bovine serum, colostral whey or an immunoglobulin containing fraction of serum or whey was usually unsatisfactory. Antiserum prepared against serum had very weak or no precipitating antibodies to SIgA. Antiserum prepared against colostral whey or an immunoglobulin fraction of such whey usually had a predominance of antibodies to IgG and only weak antibodies to IgM and SIgA. Our best polyvalent antisera, such as the one in Figure 10, have been prepared by mixing individual

rabbit antisera. Mixing one part of an antiserum prepared against the one-third saturated ammonium sulfate insoluble fraction of serum (mostly antibodies to IgG) with three parts of an unabsorbed antiserum to bovine SIgA (mostly antibodies to IgA, FSC and IgG1) and three parts of an unabsorbed antiserum to bovine serum IgM (mostly antibodies to IgM) resulted in an excellent polyvalent antiserum.

#### Discussion

The methods described for the preparation of the various bovine immunoglobulins and related proteins have been successful in our laboratory. These methods require a minimum of equipment and utilize four procedures: precipitation, gel filtration, electrophoresis and DEAE chromatography. The electrophoretic and immunochemical (immunodiffusion, immunoelectrophoresis and radial diffusion) methods employed to assay for purity are sensitive and the immunodiffusion and radial diffusion methods simple and inexpensive. The immunoelectrophoretic (Fig. 3) and disc electrophoretic (Fig. 4) patterns shown for the various preparations are diagnostic and highly reproducible.

All immunoglobulins we describe have been shown by others to be antigenically related to their human homologs (1, 20, 21, 40) except for the specific homology between subclasses of IgG. The nomenclature used for the bovine subclasses has been recently proposed by workers in the field (29). The homology of bovine FSC to human secretory component (39) is supported by immunofluorescent studies (42) and studies which demonstrate its complexing to the IgA of other species (19).

IgM prepared by gel filtration of the euglobulin fraction of serum followed by Pevikon electrophoresis is of adequate purity for almost all immunochemical and physicochemical studies. The preliminary removal of much of the  $\alpha$ -2 macroglobulin by recycling gel filtration expedites the final removal of  $\alpha$ -2 macroglobulin by preparative electrophoresis. As radial diffusion is a more sensitive technique than immunoelectrophoresis, the IgG determinants detected in the IgM preparation could result from determinants shared by  $\mu$ - and  $\gamma$ -chains rather than IgG contamination. Reaction with anti-IgG1 or anti-IgG2 could be eliminated by gel filtration of IgM in the presence of 5 M guanidine hydrochloride (GuHCl) but this could be explained by denaturation as well as by the removal of aggregated IgG. Treatment of IgM with GuHCl results in the loss of antigenicity, suggesting denaturation, and

makes the value of such a procedure questionable. A combination of preparative ultracentrifugation of euglobulins (31) followed by Pevikon (21, 38) or Geon/Pevikon (12) electrophoresis has been used successfully for the preparation of various IgM's. Recently, a detailed method employing ion-exchange chromatography has been described for the preparation of bovine colostrum IgM (23). It was our experience that better yields and cleaner preparations of IgM could be obtained from serum than from colostrum by the method we describe. The preparations of IgM from colostrum were complicated by the presence of high molecular weight polymers of IgG, some of which could be covalent (7).

IgM preparations that had been stored in a concentrated form in aqueous buffers or reconstituted from lyophilized material generally showed two precipitin lines when tested by immunodiffusion using a specific anti-IgM serum (Fig. 9E). The outermost line is believed to correspond to the 32 S polymer observed on ultracentrifugational analyses. Two precipitin lines could occasionally be seen with serum also (Fig. 9E). The presence of higher IgM polymers has been reported in the human (34).

Preparation of IgG2 and IgG1 from serum and colostrum whey, respectively, is emphasized as the easiest way to obtain preparations of high purity. IgG1 preparations from serum are sometimes contaminated with transferrin and IgG2 from normal or colostrum whey is easily contaminated with a variety of other materials, including digestion fragments and lactoferrin (6). Similar methods for the preparation of these proteins are also described elsewhere (1, 4, 20, 24, 26).

The pooling of several of the SIgA preparations described with subsequent rechromatography, was necessary to obtain preparations containing much greater than 75% SIgA. The best preparations of SIgA prepared in our laboratory have been obtained by gel filtration in the presence of GuHCl (7), and such a preparation is shown in Figure 3. Similar to IgM, losses in antigenicity, immunogenicity and alterations in carbohydrate content were encountered by the use of GuHCl. The use of acrylamide for gel filtration overcomes the carbohydrate problem. Preparations containing 75% SIgA have also been obtained by step-wise DEAE-cellulose chromatography (15). Preparations containing up to 20% IgG have been used for establishing standard curves for radial diffusion assay by correcting

for the amount of contaminating IgG (8, 9). The use of zinc ions to prepare fractions of whey or serum enriched in IgA appears to be as valid for bovine IgA as for human IgA. Our results substantiate the early findings of Gough et al. (14). The insertion of this step into the fractionation procedure (Fig. 1) eliminates much of the IgG in the WSIF prior to fractionation by gel filtration. In addition, treatment of the WSIF with  $Zn^{++}$  results in the dissociation of presumably hydrophobically bonded FSC from SIgA or IgM. This was suggested by immunoelectrophoretic analyses (Fig. 7, Wash) and was confirmed by gel filtration. The application of various zinc sulfate and gel filtration methods for the preparation of human IgA is discussed by Vaerman (40).

Preparations of IgA of the purity common in IgM and IgG preparations are difficult to obtain by any of the methods we describe, particularly when biological integrity is a strong consideration. This is especially true for bovine serum IgA. For this reason, the use of isoelectric focusing as described by Porter and Noakes (28) or the use of immuno-adsorbents as described by Zschecke et al. (43) may be the methods of choice for final purification of IgA.

All of the preparations we described are suitable for use as antigens in the production of diagnostic antisera. The results of such immunization, the procedures used and the specificity of the antisera obtained have been described.

The presence of a 7 S IgM in bovine serum has not been previously reported although such a protein has been described in the human (5, 35, 37). Unlike human 7 S IgM, the bovine protein appears to lack antigenic determinants present on 19 S IgM (Fig. 9E) (37) and precipitates with the euglobulin fraction of serum (Fig. 5) (5). These differences, however, are probably due to differences in antisera specificity and the method of euglobulin precipitation employed.

The presence of proteolytic digestion fragments of bovine IgG immunoglobulin has not been previously reported. The occurrence of human Fc and Fc' fragments is well recognized in human secretions and their presence complicates accurate quantitation of immunoglobulins by radial diffusion (39). We have also detected similar fragments in bovine serum (7) although their level is apparently too low to be a significant factor in complicating quantitative radial diffusion data (8). The multiple

radial precipitin zones obtained by some investigators studying bovine secretions (18, 22) could be the result of such fragments. The discovery of such fragments in our laboratory was prompted by the appearance of such multiple zones when radial diffusion assays were conducted using monospecific antisera. For the same reason, should 7 S IgM be abundant in certain bovine sera, quantitative data on IgM, obtained by radial diffusion, could be seriously in error.

We, as well as several other investigators (11, 17, 27, 32), have observed the presence of unidentified precipitin lines and spurs after immunoelectrophoretic analyses of serum and secretions. The data presented here on the precipitin arcs formed by IgG2 aggregates and digestion fragments (Fig. 9A), along with our observation on the differences in the immunoelectrophoretic precipitin arcs of 19 S and 7 S IgM (Fig. 9F) and our reported observations on IgA heterogeneity (7), provide a hypothesis for explaining some of these additional precipitin arcs without postulating new classes and subclasses of bovine immunoglobulins.

The partial identity reaction observed between IgM or IgA and IgG when tested with antiserum to light chains (Fig. 9B) could be accounted for by assuming that some of the light chain determinants are buried in the higher molecular weight immunoglobulins (IgM and IgA) and not available for reaction with their antibodies or by the presence of some  $\gamma$ -chain specific antibodies in the anti-L chain antiserum.

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